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Full Length Research Paper

Characterization of beneficial properties of plant growth-promoting rhizobacteria isolated from sweet potato rhizosphere

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Laboratory study was conducted to characterize the beneficial properties of plant growth-promoting rhizobacterial (PGPR) strains isolated from sweet potato rhizosphere. Fifteen rhizobacterial (PGPR) strains were screened for indole acetic acid (IAA) production with and without addition of the precursor L-tryptophan (L-TRP), phosphate-solubilizing activity, and nitrogen production, antagonistic activity against fungal pathogens, siderophore production and intrinsic antibiotic resistance. Results of the laboratory study showed that 15 rhizobacterial isolates were able to produce indole acetic acid (IAA). The concentration of IAA produced ranged from 3.84 - 13.33 mg L^{-1}. Addition of L-tryptophan (L-TRP) to the bacterial isolates increased the production of IAA ranging from 4.94 - 46.66 mg L^{-1}. Six isolates (40%) were able to solubilize insoluble phosphate as evident by production of clear zone on calcium phosphate medium. All isolates were able to grow in N-free media indicating their abilities to produce nitrogen which ranged from 0.74 - 1.32 ppm. Three of the isolates produced fluorescent pigment on agar plate indicated their abilities to produce siderophores. Four isolates were able to inhibit the fungal pathogens *Rhizoctonia* sp. and *Pythium* sp. The intrinsic antibiotic test showed that all isolates were resistant against Chloramphenicol (10 and 30 µg mL^{-1}), Streptomycin (10 µg mL^{-1}), Kanamycin (5 and 30 µg mL^{-1}), Penicillin (10 µg mL^{-1}) and Tetracyclin (30 µg mL^{-1}). The rhizobacterial isolates showed the several beneficial traits that could improve plant growth.

Key words: PGPR, indole acetic acid, phosphate-solubilization, siderophores, intrinsic antibiotic resistance.

INTRODUCTION

Beneficial effects of PGPR on plant growth have been attributed to mechanisms such as production of phyto-hormones, solubilization of phosphates, suppression of pathogens by producing antibiotics and siderophores or bacterial and fungal antagonistic activity. Many species of PGPR are able to synthesize phytohormones (Frankenberger and Arshad, 1995). The main group of phytohormones is auxin, cytokinin, gibberellin, and ethylene like substances. One of the phytohormones produced by soil microorganisms is indole-3 acetic acid (IAA) which is an important hormone for plant growth and development. The species of bacteria capable of produc-ing IAA include *Pseudomonas* sp. *Bacillus* sp. *Klebsiella* sp. *Azospirillum* sp. *Enterobacter* and *Serratia* sp. (Martens and Frankenberger, 1991; Frankenberger and Arshad, 1995). Colorimetric method is the simplest method and has long been employed for the detection of indole-3-acetic acid (IAA) produced by plants and microorganisms (De Vay et al., 1968). Different bacterial species are also able to solubilize complex inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986; Rodriguez and Fraga, 1999). Some of bacterial species such as *Pseudomonas* sp. *Bacillus* sp. *Enterobacter* sp. and *Erwinia* sp. act as antagonist inhi-

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biting growth of pathogen through production of antibiotics and siderophores (Howell et al., 1988; Thomashow and Weller, 1988). Nitrogen fixation can be carried out by several associative and free-living micro-organisms in the rhizosphere of plants and it is recognized to play an important role in nitrogen nutrition of plants (Boddey et al., 1996). Colonization of bacteria having several of the beneficial traits has been shown to promote and stimulate plant mechanisms (Sindhu et al., 1999). Exploitation of this type of bacteria to improve crop production has become important in sustainable agriculture. The following studies were conducted to screen for the activities of IAA production, phosphate solubilization, nitrogen production, siderophore production, antagonistic activity, and intrinsic antibiotic resistance by rhizobacterial strains isolated from sweet potato rhizosphere.

MATERIALS AND METHODS

Fifteen isolates were screened for the activities of IAA production, phosphate solubilization, nitrogen production, siderophore production, antagonistic activity, and intrinsic antibiotic resistance. The bacterial isolates were then identified using the Biolog Identification System (The Micro Log System Release 4.0).

Screening for IAA- producing activity

A modified colorimetric method was used for determination of IAA (Asghar et al., 2000). Pure colonies of 15 isolates obtained earlier from sweet potato rhizosphere were grown in 50 ml conical flask containing 25 mL King's B (King *et al*., 1954) broth with and without L-TRP (0.5%) solution and incubated at 28 ± 2°C for 24 h on a shaker. The cultures were then centrifuged at 4000 rpm for 20 min. One-milliliter culture supernatant was placed in a test tube and mixed with 2 mL Salkowski reagent A (2% of 0.5M $FeCl_3$ in 35% perchloric acid). After 20 - 25 min, when the color of supernatant containing IAA turned red, the color absorbance was read using a spectrophotometer at 535 nm. Each test was replicated three times. Pure IAA was used for preparing the standards of 0, 5, 10, 15, 20, 25, 30, 35, 40, and 45 mg L^{-1}.

Screening for phosphate solubilizing activity

The ability of isolates to solubilize phosphate was assessed qualitatively using Potato-Dextrose Yeast Extract Agar (PDYA) containing freshly precipitated calcium phosphate, that is 50 mL sterile 10% (wt. vol^{-1}) K_2HPO_4 and 100 mL sterile 10% ($wt.vol^{-1}$) $CaCl_2$ were added per liter sterile PDYA to produce a precipitate of $CaHPO_4$ (Katznelson and Bose, 1959). Each bacterial culture were spot inoculated in the centre of a PDYA-CaP plate (n = 3) and incubated at 28 ± 2°C for 10 days. Phosphate solubilization was assessed by measuring the clear/halo zone. The halo zone was calculated by subtracting bacterial colony diameter from the total halo zone diameter (Freitas et al., 1997). Each test was replicated three times.

Screening for nitrogen producing activity

Bacterial isolates were cultured in Nitrogen Free Broth media (Xie et al., 2003) with shaking for 24 h at 28 ± 2°C. Total nitrogen content in culture was quantified using microkjeldahl method (Bremner and Mulvaney, 1982). Approximately 1.0 mL of sample was subjected to kjeldahl digestion in a digestion flask containing concentrated sulphuric acid. The clear digest was analyzed for total N on an autoanalyser. Each test was replicated three times.

Screening for siderophore producing activity

Siderophores are low molecular weight organic molecules that have high affinity for Fe^{3+}. The bacterial cultures were streaked onto the King's B media with and without (50 μg L^{-1}) $FeCl_3$ and incubated at 28 ± 2°C for 48 h. The fluorescence pigment of the bacterial colonies and that difuse in the surrounding agar was evaluated using an ultraviolet lamp. Fluorescence pigment formed was considered as an indication of siderophore production (Teintze et al., 1981). Each test was replicated three times.

Screening for antagonistic activity

Currently there is not much information on bacterial and fungal pathogens in sweet potato. In this study, the bacterial isolates were screened for their ability to suppress growth of the common fungal pathogens; *Pythium* and *Rhizoctonia*. Potato Dextrose Agar (PDA) was used to study the antagonistic activity since both types of microorganisms (fungi and bacteria) can grow on this medium. A 5 mm mycelial plug was taken from the peripheral edge of five days old cultures of fungal pathogens and each placed at the centre of the plates. The bacterial strains were then individually streaked 2.5 cm from the mycelial plug at 4 opposite locations around the periphery of the plate and the dual culture plates were incubated at 28 ± 2°C Thereafter, the zone of inhibition (distance between the edges of the bacterial streak and the fungal mycelium measured in mm) was recorded after 3 days of growth (Deka Boruah and Dileep Kumar, 2002). Each test was replicated three times.

Intrinsic antibiotic resistance (IAR) test

IAR test was carried out to identify the bacterial sensitivity or resistance to antibiotics. IAR has also been used in the genotypic identification of bacterial species. A plate of suitable culture medium was incubated by spreading an aliquot of bacterial culture evenly across the agar surface. Filter paper discs containing different concentration of antibiotics, Chloramphenicol 10 and 30 μg mL^{-1}, Streptomycin 10 μg mL^{-1}, Kanamycin 5 and 30 μg mL^{-1}, Penicillin 10 μg $mL^{-1}$and Tetracycline 30 μg mL^{-1} were then placed on the plate. Plates were incubated at 28 ± 2°C for 3 days. The presence of inhibition zones around the discs of the different antibiotics were noted (Bauer et al., 1966). Each test was replicated three times.

Identification of the rhizobacteria isolates using biolog system

Identification of the rhizobacteria was done by using Biolog Identification System (The MicroLog System Release 4.0). The rhizobacteria cultures were tested for their gram reaction using the staining technique and oxidase reaction prior to inoculation of the Biolog-Microplates. Some rhizobacterial cultures indicate gram-negative and oxidase-positive, while the other cultures indicate gram-negative and oxidase-negative. Subsequently, based on the results of these reactions, a particular pathway of the Biolog Identification steps was followed. Bacterial suspension of each culture prepared from 24 h old cultures on Biolog Universal Growth

Table 1. Selected plant growth promoting properties of rhizobacterial isolates.

Isolates	IAA (mg L^{-1})		P Solubilization	Total N	Siderophore	Antagonistic against		Biolog
	-L-TRP	+L-TRP	(cm)	(ppm)	Production	*Rhizoctonia* sp.	*Pythium* sp. (% inhibition)	Identification
UPMSP 1	4.19 de	4.97h	-	0.83d	-			ND
UPMSP 2	3.84e	27.25c	-	1.33a	-			*Pseudomonas corrugate*
UPMSP 3	8.37c	10.72g	1.85a	0.75d	+		+ (29.2)	*Serratia ficaria*
UPMSP 5	9.76b	15.94f	-	1.24a	-			ND
UPMSP 6	4.45de	17.33ef	1.07bc	1.17ab	-	+ (22.8)		ND
UPMSP 8	7.50c	16.11f	-	1.03bc	-			ND
UPMSP 9	5.15de	46.66a	1.23b	0.90cd	-			*Klebsiella terrigena*
UPMSP 10	11.07b	45.09a	2.03a	1.21ab	-		+ (24.8)	*Erwinia cypripedii*
UPMSP 11	4.10e	5.32h	-	1.16ab	-			ND
UPMSP 12	4.36de	21.95d	-	1.15ab	-			*Acinetobacter radioresistens*
UPMSP 13	5.06de	32.13b	-	0.90cd	+			*Pseudomonas maculicola*
UPMSP 15	13.33a	19.68de	-	1.30a	-			ND
UPMSP 16	9.93b	10.45g	-	0.88cd	-			ND
UPMSP 18	10.98b	30.99b	1.20b	1.16ab	-			*Paenibacillus pabuli*
UPMSP 20	5.50d	8.02gh	0.86c	1.15ab	+	+ (27.2)		*Pseudomonas fuscovaginae*

Note: Means in column followed with same letter (s) are not significantly different (P > 0.05)
ND: Not determined.

media (BUG) and mixed in specific fluid. Turbidity of bacterial suspension was adjusted to 52% transmittance for the cultures with the oxidase-positive reaction, and 61% transmittance for those with the oxidase-negative reaction. The suspension was then inoculated into the wells of Biolog ID Microplates by putting 140 µL of the suspension in each well. The microplates were incuba-ted at 30°C for 24 h. The results were read with the Biolog MicroStation reader and ML3 software. The reactions in the wells that turned purple which considered as positive while clear wells considered negative. The control well (well number 1) was clear indicating no reaction.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS, version 8.2, 2006) for means comparison using Tukey's Studentized Range (HSD) test at p = 0.05.

RESULTS

IAA production

Results showed that the 15 rhizobacterial isolates were able to produce IAA (Table 1). The concentration of IAA produced by the rhizobacterial isolates ranged between 3.84 - 13.33 mg L^{-1}. Five of the isolates (UPMSP15, UPMSP16, UPMSP18, UPMSP 5 and UPMSP10) were able to produce high IAA while two isolates (UPMSP2 and UPMSP11) produced very low amount of IAA in the absence of L-TRP.

Higher IAA production was observed in the presence of precursor L-TRP. The concentration of IAA produced ranged between 4.97 to 46.66 mg L^{-1}. There was significant difference in the concentration of IAA produced among the isolates. *Klebsiella* UPMSP9seemed to produce high amount of IAA while isolate UPMSP1 produced very low amount of IAA in culture medium (Table 1). The effect of L-TRP on IAA differed between bacteria. *Klebsiella* UPMSP9 showed highest response to L-TRP with a nine fold increase in IAA. Not all isolates responded to the precursor application as three of the Isolates (UPMSP1, UPMSP11 and UPMSP16) showed no significant change in IAA with L-TRP addition.

Phosphate-solubilizing activity

The results showed that, six out of 15 rhizobacterial isolates were able to form clear zone around the colonies on PDYA, an indication of calcium phosphate solubilization (Table 1). Highest clear zone of 2.03 cm was observed in *Erwinia* UPMSP10 and the smallest was formed by *Pseudomonas* UPMSP20 with diameter of 0.86 cm.

Total nitrogen production

Results showed that the 15 rhizobacterial isolates were able to grow in N Free Broth and increased the total N in the growth culture (Table 1). The amount of N produced differed among isolates. *Pseudomonas* UPMSP2, UPMSP5, and UPMSP15 isolates produced higher amount of N (1.24 - 1.32 ppm), while *Pseudomonas* UPMSP3 and UPMSP1 isolates produced very low amount of N (0.74 - 0.83 ppm).

Siderophore production

The production of siderophore was recognized by the distinct yellow-green fluorescent pigment produced by the bacteria on the medium. Fluorescent pigment could be produced on the low Fe medium, as it has a high affinity Fe^{3+} chelator (Teintze et al., 1981). Only three of the isolates *Serratia* UPMSP3, *Pseudomonas* UPMSP13 and *Pseudomonas* UPMSP20 were able to produce siderophores as shown by fluorescent pigment on King's B medium without Fe^{3+} (Table 1).

Test for antagonistic activity of bacteria against pathogen

Out of the 15 isolates, only four isolates produced zones of inhibition against the fungal pathogens. Two of the isolates, UPMSP6 and *Pseudomonas* UPMSP20 showed the antagonistic activity against *Rhizoctonia* sp. with 22.8 and 27.2% inhibition respectively. The other two isolates, *Serratia* UPMSP3 and *Erwinia* UPMSP10 showed antagonistic against *Pythium* sp. with 29.2 and 24.8% inhibition, respectively (Table 1).

Identification of the rhizobacteria isolates using biolog system

The isolates used in the study were identified as *Pseudomonas corrugate* (UPMSP2), *Serratia ficaria* (UPMSP3), *Klebsiella terrigena* (UPMSP9), *Erwinia cypripedii* (UPMSP10), *Acinetobacter radioresistens* (UPMSP12), *Pseudomonas maculicola* (UPMSP13), *Paenibacillus pabuli* (UPMSP18), and *Pseudomonas fuscovaginae* (UPMSP20) (Table 1). Isolate UPMSP11 was unable to be identified using Biolog System and the other bacterial isolates were found unable to grow after successive subcultures.

Intrinsic antibiotic resistance (IAR) test

All the isolates tested were resistant against Kanamycin (5 µg mL^{-1}), Penicillin (10 µg mL^{-1}), Tetracycline (30 µg mL^{-1}) and Chloramphenicol (10, 30 µg mL^{-1}) (Table 2). Three of the isolates (*Acinetobacter* UPMSP12, *Pseudomonas* UPMSP13, and *Paenibacillus* UPMSP18) showed sensitivity to higher dose of Kanamycin (30 µg mL^{-1}) and *Acinetobacter* UPMSP12 to Streptomycin (10 µg mL^{-1}).

DISCUSSION

The present study clearly revealed that all isolates tested in this study had the ability to produce IAA and consequently, considered as IAA producing rhizobacteria. Most studies have shown that IAA biosynthesis is greatly influenced by L-TRP precursor. L-TRP is believed to be the primary precursor for formation of IAA in several microorganisms (Frankenberger and Arshad, 1995). Addition of L-TRP (an auxin precursor) to the media increased the auxin production by several fold.

The concentration of IAA produced varied between bacterial strains. UPMSP 15 produced the highest IAA (13.33 mg L^{-1}) even without addition of precursor indicating that bacteria were actively involved in the synthesis of IAA in pure culture. Other studies have shown the ability of *Pseudomonas* and *Acinotobacter* species isolated from wheat and rye rhizosphere to produce lower IAA ranging from 0.01 - 3.98 mg L^{-1} (Leinho and Vacek, 1994). Asghar et al. (2000) showed that hundred bacterial isolates from rapeseed plant were able to produced IAA in the absence of precursor L-TRP and the highest concentration of IAA produced by one of the isolates was 11.40 mg L^{-1}.

The production of IAA could also be influenced by other factors, such as culture and medium conditions, species or strains of rhizobacteria, growth stage and availability of substrates (Frankenberger and Arshad, 1995). Most of the isolates required L-TRP precursor for IAA production. Three of the isolates *Klebsiella* UPMSP9, *Pseudomonas* UPMSP2 and *Pseudomonas* UPMSP13 showed 9, 7 and 6-fold increases respectively, in IAA production when grown in media with L-TRP. Also, other isolates, UPMSP 10, UPMSP 12 and UPMSP 18, showed between 4 and 3-fold. This shows that these strains probably synthesized IAA through TRP pathways. Several different IAA biosynthesis pathways are used by the bacteria, and a

Table 2. Intrinsic antibiotic resistance (IAR) test of rhizobacterial isolates

Isolates	Kanamycin 5 µg mL^{-1}	Kanamycin 30 µg mL^{-1}	Penicillin 10 µg mL^{-1}	Streptomycin 10 µg mL^{-1}	Tetracycline 30 µg mL^{-1}	Chloamphenicol 10 µg mL^{-1}	Chloamphenicol 30 µg mL^{-1}
UPMSP 2	(+)	(+)	(+)	(+)	(+)	(+)	(+)
UPMSP 3	(+)	(+)	(+)	(+)	(+)	(+)	(+)
UPMSP 9	(+)	(+)	(+)	(+)	(+)	(+)	(+)
UPMSP 10	(+)	(+)	(+)	(+)	(+)	(+)	(+)
UPMSP 11	(+)	(+)	(+)	(+)	(+)	(+)	(+)
UPMSP 12	(+)	(-)	(+)	(-)	(+)	(+)	(+)
UPMSP 13	(+)	(-)	(+)	(+)	(+)	(+)	(+)
UPMSP 18	(+)	(-)	(+)	(+)	(+)	(+)	(+)
UPMSP 20	(+)	(+)	(+)	(+)	(+)	(+)	(+)

Symbols (+): Resistant, (-): Sensitive.

single bacterial strain sometime containing more than one pathway (Patten and Glick, 1996, 2002). Asghar et al. (2000) had shown that the high concentration of IAA (111.50 mg L^{-1}) was produced by plant growth-promoting rhizobacteria S88 when L-TRP was added in the medium. In natural condition, plant roots excrete organic compounds including L-TRP which can then be utilized by the rhizobacteria for IAA biosynthesis (Frankenberger and Arshad, 1995).

Six of the rhizobacterial isolates showed the ability to solubilize complex calcium phosphate. Phosphate solubilization by rhizobacterial isolates has been shown to be related to the production of organic acids such as formic, acetic, propionic, lactic, glycolic, fumaric and succinic acids (Rodriguez and Fraga, 1999 and Kucey, 1983). The production of organic acids results in a decrease in soil pH, producing H^+ which replaces the Ca^{2+} and release HPO_4^{2-} to the soil solution. Besides changes in pH, chelation by organic acids which bind metal ions such as Fe, Al, and Ca bring about phosphate into soil solution. In tropical soil, the low pH influences solubilization of phosphate by rhizobacteria. Soil inoculation with phosphate-solubilizing bacteria has been shown to improve solubilization of fixed soil P and applied phosphates resulting in higher crop yields (Freitas et al., 1997; Nautiyal, 1999). Several phosphate-solubilizing rhizobacteria could also promote plant growth by rendering phosphate into solution more than they need for their metabolism, and the surplus can be absorbed by plant (Kloepper et al., 1989).

Most of the isolates were able to grow on N-Free Broth and increased the total N in the growth culture. This presumptive test indicated the N_2 fixing activity of the isolates. More direct methods need to be conducted to confirm the N_2 fixing activity. Nitrogen fixation carried out by associative and free-living microorganisms in the rhizosphere of plants has been recognized to play an important role in nitrogen nutrition of plants (Boddey et al., 1996; Zuberer, 1998). Various bacteria genera such as *Azospirillum, Klebsiella*, *Enterobacter, Pseudomonas, Erwinia, Bacillus* appear to be frequent colonizer of crops. Dobereiner (1997) has shown that associative diazotrophic microorganisms could contribute at least 20 - 40% of the plant N requirement of several non leguminous crops through N_2 fixation process. *Azospirillum* and rhizobacteria inoculation could contribute a substantial amount (25 - 50% in oil palm) of the total plant N requirement through BNF process (Amir et al., 2001).

Out of the 15 rhizobacterial isolates studied, only 4 were found to be antagonistic at varying degree to the soil borne fungal pathogens. UPMSP6 and *Pseudomonas* UPMSP20 found to inhibit the growth of *Rhizoctonia* sp while *Serratia* UPMSP3 and *Erwinia* UPMSP10 inhibited the growth of *Pythium* sp. Similarly, Safiyazov et al., (1995) reported that bacterial isolate was effective in controlling *Rhizoctonia* sp. on cotton giving an inhibition zone. *Rhizoctonia and Pythium* have been known to be agronomically important plant disease causing soil microorganisms. They have also been found in root rot disease of sweet potato plant (O' Sullivan *et al.,* 1997). Other studies have shown the beneficial effect of plant growth promoting rhizobacteria inhibiting the growth of *Rhizoctonia* sp. *Pythium* sp. and *Phyto-*

pthora sp. in vitro (Park et al., 1995).

The potential biological activity of the isolates showed that *Serratia* UPMSP3, *Pseudomonas* UPMSP13 and *Pseudomonas* UPMSP20 produced fluorescent pigment on KB medium indicating presence of siderophore. Previous studies have also reported that *Pseudomonas* sp. are known to produce pyoverdines and pseudobactins, which can be detected by their yellow-green colour fluorescence under ultraviolet light when grown on iron deficient medium (Buysens et al., 1996). Besides the fluorescent pseudomonads some non-fluorescent compounds also secreted siderophore, which is important in the survival of the bacterium. Siderophores had been shown to be involved in the suppression of various phytopathogenic fungi (Elad and Baker, 1985; Thomashow and Weller, 1990; Loper, 1988).

Six rhizobacterial isolates were intrinsically resistant to the antibiotics tested and three of the bacteria *Acinetobacter* UPMSP12, *Pseudomonas* UPMSP13, and *Paenibacillus* UPMSP18 were very sensitive to Kanamycin 30 µg mL^{-1}. Kanamycin, Streptomycin, Tetracycline, Chloramphenicol are some of the antibiotic produced by actinomycetes especially *Streptomyces* species, while Penicillin is produced by the fungi. Most prominent actinomycetes were originally obtained from soil (Alexander, 1977). Productions of antibiotics which are low molecular weight organic compounds are deleterious to the growth or other metabolic activities of other microorganisms (Fravel, 1988). The antibiotic production is an important mechanism of biological control where it inhibits cell wall synthesis of bacteria, thus inhibiting their growth (Subba Rao, 1999). Resistant of PGPR to several antibiotics might have an ecological advantage of survival in the rhizosphere when they are introduced as incoulum. Survival of inoculant in soil leads to bacterial colonization on roots and expression of their beneficial effect.

Conclusion

The study indicates that there are several beneficial traits in rhizobacterial strains that could improve plant growth. Two isolates *Klebsiella* UPMSP9 and *Erwinia* UPMSP10 possess the ability to produce high IAA with L-TRP, solubilize phosphate, produce N in culture and resistance to antibiotic such as Kanamycin (5, 30 µg mL^{-1}), Penicillin (10 µg mL^{-1}), Streptomycin (10 µg mL^{-1}), Tetracycline (30 µg mL^{-1}) and Chloramphenicol (10, 30 µg mL^{-1}). These isolates have the potential to be used as inoculant for plant growth improvement.

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